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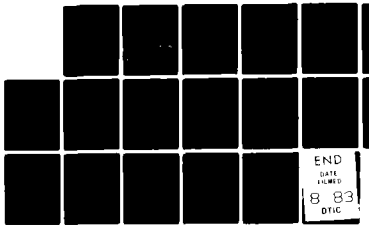

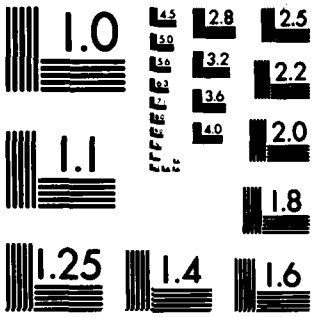

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

In this paper are described and evaluated several new tools of potential use in clinical chemistry. The first, intended to minimize required sample volumes, is a device with which a total sample volume of 1 μL can be dispensed in the form of 1000 identical aliquots. Any number of such nanoliter aliquots can be taken if larger samples are needed. The second new tool is one for detecting anions or cations separated by ion chromatography. Unlike conventional conductometric detectors used in ion chromatography, the new system offers potential sensitivities in the sub-ng/L range and useful operating ranges up to

microgram

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100 mg/L. The third tool is a scheme for background correction in atomic absorption spectrometry; the new technique requires no special auxiliary sources or double-beam optics. Finally, fluorescence time-decay curves and fluorescence lifetimes are shown to be able to overcome the effects of diffusional quenching and scattering resulting from turbidity of solutions in clinical fluorometry.

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NEW TECHNIQUES AND TOOLS FOR CLINICAL CHEMISTRY

by

Gary M. Hieftje

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Indiana University
Department of Chemistry
Bloomington, Indiana 47405

1 July 1983

Clinical chemistry and clinical analysis can benefit greatly from advances in chemical instrumentation.

It is therefore important for clinical chemists and others to be aware of new developments in instrumentation.

In this paper are described several such advanced tools, only one of which is yet commercially available.

A Nanoliter Sample Dispenser

The ability to dispense small volumes of sample easily and accurately is important in clinical chemistry. The smaller the sample that can be assayed, the lower the cost and the greater the number of repetitive (or different) assays that can be performed on a specimen. Moreover, because each microsample can be treated individually, it enables an instrument to be optimized between aliquots by a control computer; work is underway in our laboratory to develop semi-intelligent instruments which can optimize themselves and produce interference-free, nearly ideal assays on any sample, regardless of its origin or whether the instrument has "seen" the sample before.

The new microsampler to be described here can use total sample volumes as small as 1 μL and dispense even such a small specimen into as many as 1000 identical 1-nl aliquots. Essentially, this sampler operates by repetetitively withdrawing a glass needle from a bulk liquid, nanoliter-volume aliquots (droplets) being formed as illustrated in Figure 1.

Details of the operation of this new device appear elsewhere (1). In summary, the present device can produce nanoliter droplets with a precision (CV) of better than 1% and at production rates as high as 1000 droplets per second. The surface tension and viscosity of the

sampled liquid are important in determining droplet size, but uniform droplets can be reproducibly formed over a wide range of temperatures, viscosities, and surface tensions. The necessary electronic circuitry and mechanical hardware are relatively simple (1), and the system can be conveniently coupled to a laboratory microcomputer for automated sampling procedures.

Not surprisingly, the precision of microsampling increases with the number of individual aliquots taken. Under ideal conditions - i.e. where the variation among droplets is completely random - it should be possible to improve sampling precision by employing multiple aliquots, the precision being improved in proportion to the square root of the number of droplets dispensed per sample (1).

There are many potential applications of this sampler to clinical chemistry. For example, the system has already been shown to be extremely useful in analyses by electrothermal atomization atomic absorption spectrometry (2), where it was shown that the precision with which the sample was applied to the carbon furnace was improved dramatically. Moreover, because sample solutions are dispensed and fall by gravity into the carbon furnace in the form of tiny droplets, the resulting residue consists of tiny crystallites, much smaller than those produced by a conventional micropipette. As a result, samples were volatilized in the carbon furnace more reproducibly and "occlusion-based" interferences were far less severe. Such interferences arise frequently when a micropipette is used because the desired elements are incorporated into large crystallites in the sample residue. Finally, the system is especially convenient in this particular application because samples can be placed in the carbon furnace receptacle in convenient, multiple-droplet aliquots, the total volume of which is adjustable under computer control.

Thus, the atomic-absorption calibration curve can be constructed on the basis of sample mass, which is proportional to the number of droplets, rather than concentration. Accordingly, the calibration curve can be constructed from a single standard solution, a capability which simplifies routine determinations, especially for unusual samples.

In another application, the sampler was used as an automatic titrator for microliter-size samples (3). As a microtitrator, this new device was able to analyze samples in volumes smaller than 20 μL with a precision (CV) approaching 0.1%. In a current extension of this work, the system is used in a "pH stat" mode to measure (e.g.) enzyme activity and substrate concentration (4).

Additional applications include the dispensing of precious enzyme solutions, of which only tiny amounts are needed or available. Similarly, clinical samples such as cerebrospinal fluid from infants would be optimally handled with the new apparatus.

Replacement Ion Chromatography

Ion chromatography is a relatively new analytical technique which is based on conventional ion-exchange chromatography, but reconfigured for high sensitivity and precision. In ion chromatography, the specific ions of interest (anions or cations) are separated by conventional ion-exchange techniques. During this chromatographic process, the ions to be measured are eluted sequentially from an appropriate packed column by an appropriate ion (often hydrogen or hydroxide) that can displace them from that column. The eluted ions then are usually and simply detected with a conductometric cell.

The sensitivity of detection can be improved by decreasing the background conductance of the eluting solvent before the detection

step. This is done by passing the eluting liquid through a second column, termed the "suppressor" column, which serves to neutralize the eluting solvent or render it relatively nonconductive. For example, in cation separations, H^+ is often used as an eluting agent. The resulting acid then is eluted continuously from the column, and it generates a relatively high background conductance against which must appear the small conductance change produced by the ions of interest. To decrease this background conductance, the entire solution is passed through an anion-exchange column that initially is in the hydroxide form. Anions eluting with the original hydrogen ions then displace OH^-, which combines with the residual H^+ to form water, which is relatively nonconductive. The result is that eluting ions of interest are measured against a very low background.

Unfortunately, in ion chromatographs such as the one just described, conductivity is measured. Such measurements have limited sensitivity, require separate calibration curves for each ion of interest, and temperature changes cause drift. We have developed a new technique, which we term "replacement ion chromatography", to overcome these limitations.

In replacement ion chromatography (5), a conventional ion-exchange column and "suppressor" column are used just as in ordinary ion chromatography. Therefore, ions elute at the expected times and can be identified on the basis of their retention times. However, the eluting ions of interest are all stoichiometrically replaced in a third column by an ion that can be more sensitively detected.

For example, in the case of the cation analysis mentioned above, cations would elute sequentially, in expected order and at expected times, in nearly pure water from the conventional ion

chromatography column combination. This flowing solution would then enter a third cation-exchange column, in the lithium form. Thus, the eluting cations would quantitatively displace lithium, a rather easily displaced ion, which then is detected. In this example, lithium was selected, partly because it can be easily displaced by other ions, but also because, by flame photometry, it is one of the most easily and sensitively (10^{-8}mol/L) detected ions. Moreover, because all sample ions are replaced by lithium, a calibration curve constructed for lithium can be used for all other ions as well. Finally, flame photometry is far less sensitive to temperature variations than is a conductivity cell.

In the first description of this apparatus (6), we have shown that the linearity of the working curve corresponds to that expected of flame photometry (e.g. Figure 2). However, detection limits are as yet less impressive than one might hope - principally because of lithium "bleed" from the replacement column which causes undesirably high background in the flame photometer and consequently poorer detection limits. This problem is exacerbated by the turbulent and therefore rather noisy flame used in our preliminary investigations. With it detection limits for this procedure, which on a molar basis are obviously the same for all ions, are on the order of 10^{-6}mol/L.

The calibration surve in Figure 2 is constructed on the basis of the time-integrated Li^+ emission signal, to enable the same calibration curve to be used for all cations and anions (see below). Of course, if the feature is not required, peak height would also be a suitable vertical axis.

Determination of anions (cf. Figure 2) requires no further additions to the system: the same flame photometer, the same replacement ion (lithium), and the same replacement column can be

used for the determination of anions or cations. This capability exists because each eluting anion (during anion analysis) must have associated with it a counter cation. In our implementation of anion replacement-ion chromatography, this counter cation is lithium, which is detected with the same flame photometric apparatus used for cation replacement-ion chromatography.

The applicability of this technique to the determination of anions is particularly important in clinical chemistry. Although cations (metals, in particular) can be determined by atomic emission or absorption methods, there is no comparably simple method for anion determination. Instead, most anions must be determined individually by indirect or traditional chemical methods, techniques that in many cases are relatively time-consuming and error-prone. In contrast, anion replacement-ion chromatography should provide sub-μg/L detection-limit capability for both anions and cations. Figure 3 shows a chromatogram for monovalent anions, and shows how quickly such determinations might be made. The peak tailing evident in Figure 3 is not uncommon in ion chromatography and is not greatly worsened by the third column used in the new system.

Future work in the replacement-ion chromatography of anions and cations will involve the use of different replacement ions and alternative detection techniques. Fluorimetry is a particularly promising and highly sensitive method for measuring certain ion species. Unfortunately, most ions of clinical interest do not fluoresce strongly and cannot be so measured. However, if a suitable fluorescing ion were used in replacement-ion chromatography as Li^+ was used above, each ion of interest would be stoichiometrically converted to the fluorescing species and could therefore be determined at equivalently high sensitivity. We

currently are working on this and other methods of detection.

Background Correction in Atomic Absorption Spectrometry

The low concentration of metals and other cations in most clinical samples makes electrothermal atomic absorption spectrometry the technique of choice for elemental analysis. Unfortunately, the sample matrix frequently causes problems. In particular, smoke produced by in situ ashing of clinical samples and the possibility that some elements such as lead can be partly or completely volatilized during this ashing process necessitates the use of background-correcting methods. Without such methods, the "smoke" evolved from the sample matrix can obscure the volatilizing elements and cause erroneously high measurements.

Background correction with atomic absorption spectrometry is now a well-developed art. For background correction, two widely used techniques are currently available, one based on the use of a deuterium arc source, and the other on the "Zeeman" method. Both of these approaches have drawbacks. The former (7) requires a separate background-correction source (the deuterium lamp itself), necessitates double-beam optics, and is not particularly useful in the visible portion of the spectrum, where many elements of clinical interest appear. The latter requires fairly sophisticated instrumentation, a relatively expensive magnet to produce the requisite "Zeeman splitting" (8,9), and can yield double-valued working curves.

A new method for background correction (10) overcomes many of these limitations. Unlike the deuterium arc arrangement, it requires no separate source, but uses for background correction the hollow-cathode lamp that is ordinarily required for atomic-absorption measurements. Moreover, it requires no double-beam optics, no exotic instrumentation, no magnet, and produces working curves which

do not bend over upon themselves. Importantly, this new technique is less expensive than others currently in use and a commercial version of the instrument has just been announced (11).

This new technique for background correction is based upon the broadening of a spectral line emitted from a hollow-cathode lamp when that lamp is operated under high currents. At low currents, the elemental emission line from a hollow-cathode lamp is extremely narrow, a feature that is important in atomic absorption spectrometry if linear working curves are to be obtained. In contrast, operation of the hollow-cathode lamp at high currents broadens the emitted spectral line and this difference in line width permits background subtraction.

Because atomic absorption spectral lines are themselves relatively narrow, they can absorb almost completely the narrow emission line from a hollow-cathode lamp. In contrast, a broadened hollow-cathode emission line can be absorbed only partly by atoms in a flame or carbon furnace. In essence, atomic absorption removes only the center of the broadened emission line. As a result, atomic absorbance values calculated for the narrow hollow-cathode emission line(obtained at low current) differ greatly from those calculated for the broadened emission line (obtained at high current). The difference between the two values is therefore proportional to atom (or element) concentration.

In contrast, a broad-band (molecular) absorber or smoke (which scatters hollow-cathode radiation) will affect equally the narrow or broadened lamp emission line. Therefore, absorbance values calculated for the two cases would be the same and subtracting them would completely remove any effects of background

absorption or broad-band scattering.

This new system for background correction depends upon operating a hollow-cathode lamp successively at two different currents. The first, a low-current step, yields an emission line that is very narrow; the resulting calculated absorbance then contains contributions from both atomic and background features. At the end of the low-current step, a high-current (up to 0.5 A) pulse occurs, during which the hollow-cathode line is broadened substantially, so that an absorbance calculated during that period registers principally background features. When the absorbance values taken during the low-current and high-current cycles are subtracted, the difference is a value that is proportional to atomic absorbance but independent of background features. Importantly, the high-current pulse is brief enough and the average lamp current is low enough that hollow-cathode lamps used with this new technique last as long as those used in conventional atomic absorption measurements (10,11).

Working curves obtained during the low-current and high-current cycles of the hollow-cathode lamp are shown in Figure 4. The difference between them, labeled "DELTA", is the background-independent value.

That this new background-correction technique works effectively can be seen from Figure 5, which shows an extreme case. The salt-based sample used in Figure 5 poses an even greater background correction problem than those commonly encountered in clinical chemistry. The salt matrix produces an inordinate amount of smoke during sample ashing and atomization and makes accurate atomic-absorption determinations extremely difficult. Yet, the cadmium determination that is portrayed in Figure 5 shows no

error attributable to background.

In this new system, background is corrected very near the atomic line of interest, so that spectrally structured backgrounds can still be overcome. Moreover, extremely high background levels can be tolerated; Figure 5 shows that background absorbance values up to A = 3 can be overcome. Details of this background-correction technique will be published elsewhere (10).

Time Resolution in Clinical Fluorimetry

Fluorimetry is among the most sensitive of analytical techniques and thus enjoys a place of importance in clinical assays. Unfortunately, its wider use in clinical chemistry is restricted in part by errors to which such measurements are susceptible. Common sources of error in clinical fluorimetry include scattering of incident light and the effects of quenching of the desired fluorescing material.

Errors caused by scattering result from turbidity of common clinical samples. Turbidity can often be decreased by filtering, but filtration increases the likelihood of sample loss by adsorption or by inclusion in the discarded particulate material.

Quenching-caused errors in clinical fluorimetry arise from the uncontrolled deactivation of excited molecules which would otherwise fluoresce. Because such collisional quenching depends on sample matrix, and can be induced by environmental factors (oxygen is a particularly bothersome culprit), heroic means are usually used in fluorimetric procedures to rigorously exclude quenchers from the sample material or to control the concentration of quenching agents. For example, removal of oxygen usually requires bubbling an oxygen-free gas (usually nitrogen) through

the sample solution.

Obviously, fluorimetric procedures that don't have these problems would be welcome. Time-resolved fluorimetry provides just these capabilities.

The ability to separate fluorescence from scattered radiation arises from the temporal characteristics of the fluorescence and scattering processes themselves. Whereas scattering is an instantaneous process, fluorescence events have an intrinsic lifetime that is characteristic of the particular species under investigation. As a result, the time profile of scattering follows that of the exciting light source, while fluorescence, like phosphorescence, briefly persists after the exciting light source has died. This situation is depicted in Figure 6.

In Figure 6, it is assumed that a pulsed excitation source is used whose time behaviour is reflected in that of the scattered radiation (Figure 6C). By comparison, the intrinsic lifetime of fluorescence causes it to be delayed somewhat from scattered radiation (Figure 6B). Therefore, it is possible to select a time for viewing fluorescence at which the scattering has diminished nearly to zero; such a time is illustrated by the position of the "photodiode gate" in Figure 6.

The "photodiode gate" depicted in Figure 6C might be of electronic or optical origin or could result from a combination of the two. In our particular implementation (12), the gate is derived from a combination of optical and electronic elements and provides unusually high time resolution. As a result, even fluorescing agents having relatively short lifetimes can be separated from scattered light.

As an extreme illustration of this capability, Figure 7

demonstrates that even resonance fluorescence can be separated from scattered radiation, "resonance fluorescence" being that which occurs at the same wavelength of the exciting light and therefore also at the same wavelength as scattered radiation. Thus, it is not possible to separate scattering from resonance fluorescence on the basis of a spectral shift. Yet, even in this difficult situation, time resolution enables a fluorescence signal (atomic fluorescence, here) to be essentially immune to the effects of scattered radiation.

The particular instrument employed in our work uses an electronic device, a microwave mixer, to perform the "gating" function depicted in Figure 6. In turn, the "gating" waveform (bottom of Figure 6) is derived from a photodetector that directly monitors the light source. This detector's output then multiplies the combination fluorescence/scattering signal in the microwave mixer to yield a sampled version of the original waveform, but one which is free from scattering interference.

A slight modification of the apparatus just described enables it to determine completely the fluorescence lifetime of a desired chemical species (13). In turn, knowledge of that lifetime can be used to overcome the deleterious effects of collisional quenching in clinical assays. The basis for this capability resides in the congruency between the effects which such quenching has on the intensity of fluorescence from a desired species and the lifetime of that fluorescence.

In particular, under clinical conditions it is often assumed that fluorescence (F) is directly proportional to concentration (C) of the sought-for substance.

$$F = kC \qquad (1)$$

However, this simple relationship overlooks the effects of quenching. To incorporate those effects into equation 1 requires the addition of a factor termed "quantum efficiency" (ϕ). In effect, the quantum efficiency is the fraction of excited molecules that actually fluoresce. When quantum efficiency is included, equation 1 becomes

$$F = k\phi C \qquad (2)$$

Conveniently, the quantum efficiency (ϕ) can be expressed as the ratio of the actual lifetime of the substance under investigation (τ) to the species' intrinsic radiative lifetime (τ_o).

$$\phi = \frac{\tau}{\tau_o} \qquad (3)$$

Combining equations 2 and 3 then yields

$$F = k \left(\frac{\tau}{\tau_o}\right) C. \qquad (4)$$

Equation 4 can be rearranged by recognizing that the intrinsic lifetime (τ_o) is a constant, like k.

$$\frac{F}{\tau} = k'C \qquad (5)$$

Therefore, one could obtain a quantity that is independent of quantum efficiency (and therefore independent of quenching effects) simply by dividing the fluorescence intensity of the sought-for substance by its fluorescence lifetime.

The ability of this approach to correct for quantum efficiency (quenching) variations can be seen from Figure 8 (14). Chloride ion is known to affect strongly the fluorescence of quinine. From the lower curve in Figure 8, it is apparent that an unknown chloride ion concentration would cause dramatic errors in a quinine assay.

However, if the measured quinine fluorescence from each sample is divided by its fluorescence lifetime, the upper curve in Figure 8 results. Obviously, almost complete immunity to quenching errors can be realized.

Although few laboratories are currently equipped to measure fluorescence on a time-resolved basis, apparatus is already commercially available for just such measurements, and future developments (13) should reduce the cost of the necessary instrumentation.

Conclusion

This brief sampling should indicate the diversity and power of new chemical instrumentation that might ultimately be applied in the clinical laboratory. Clinical chemists need to be aware of such developments.

Acknowledgement

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FIGURE LEGENDS

Fig.1 Photomicrographs of nanoliter sampling device in action.

1) Glass needle poised for insertion into dyed liquid held within a 3-mm inside-diameter bulk-liquid reservoir;
2) Needle shown fully driven into liquid;
3) Needle withdrawing from bulk sample pulling with it a liquid filament;
4) Filament detaches from needle;
5) Then from bulk liquid;
6) Filament collapses into a nanoliter sample droplet.

Fig.2 Universal calibration curve for replacement-ion chromatography with flame photometric detection of lithium *, F^-; o, Cl^-; , Br^-.

Fig.3 Detection of halides (1 mmol/L each) by replacement ion chromatography. Eluent 2.0 mmol/L Na_2CO_3, pH 10.5; flow rate 1.8 mL/min. Volume injected: 0.100 mL. Lithium emission measured at 670 nm.

Fig.4 Atomic-absorption working curves for Ni in a N_2O-C_2H_2 flame obtained under conditions of high (bottom curve) and low (top curve) current operation of a hollow-cathode lamp. Difference between curves, labeled "DELTA", is used for background correction.

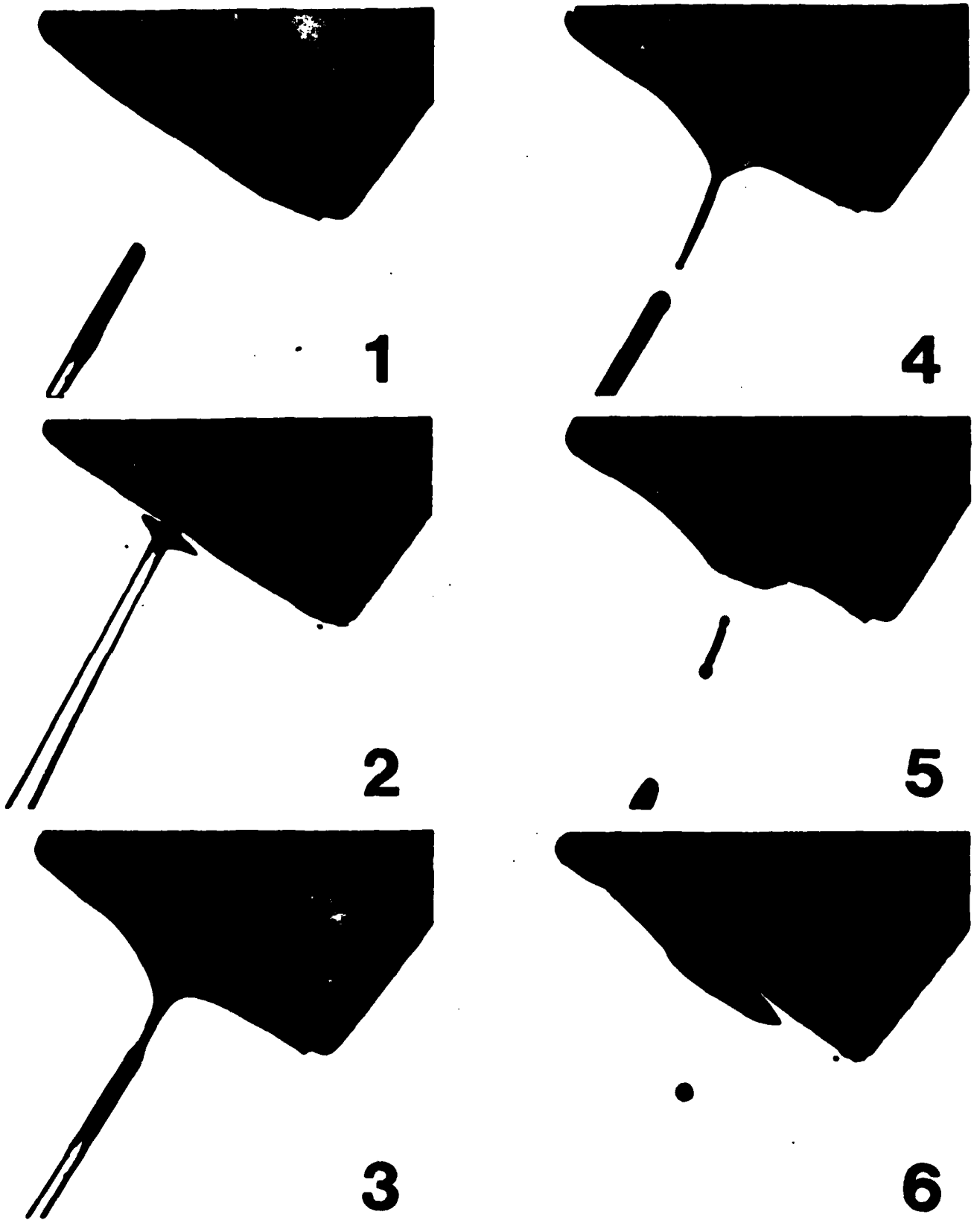
Fig.5 Atomization curves for 50 pg Cd in 200 µg sodium chloride matrix, obtained with a carbon furnace. The upper curve, labeled 1, shows the erroneously large absorbance signal generated by the salt matrix when no background correction is employed. The lower curve (2) reveals the true temporal profile of Cd absorbance,

obtained with the new background-correction method.

Fig.6 Fluorescence can be separated from potentially interfering scattered radiation by use of time-gating techniques. See text for discussion.

Fig.7 Reduction of the effect of scattered radiation in the resonance fluorescence measurement of sodium in an air-acetylene flame. A, conventional observation; B, time-gated sampling of fluorescence 1.6 ns after Na excitation.

Fig.8 The effects of collisional quenching can be overcome in clinical fluorimetry by dividing the measured fluorescence by the fluorescence decay time. Uncorrected (o) quinine bisulfate fluorescence is strongly quenched by chloride ion whereas the lifetime-corrected value (□) is nearly independent of chloride.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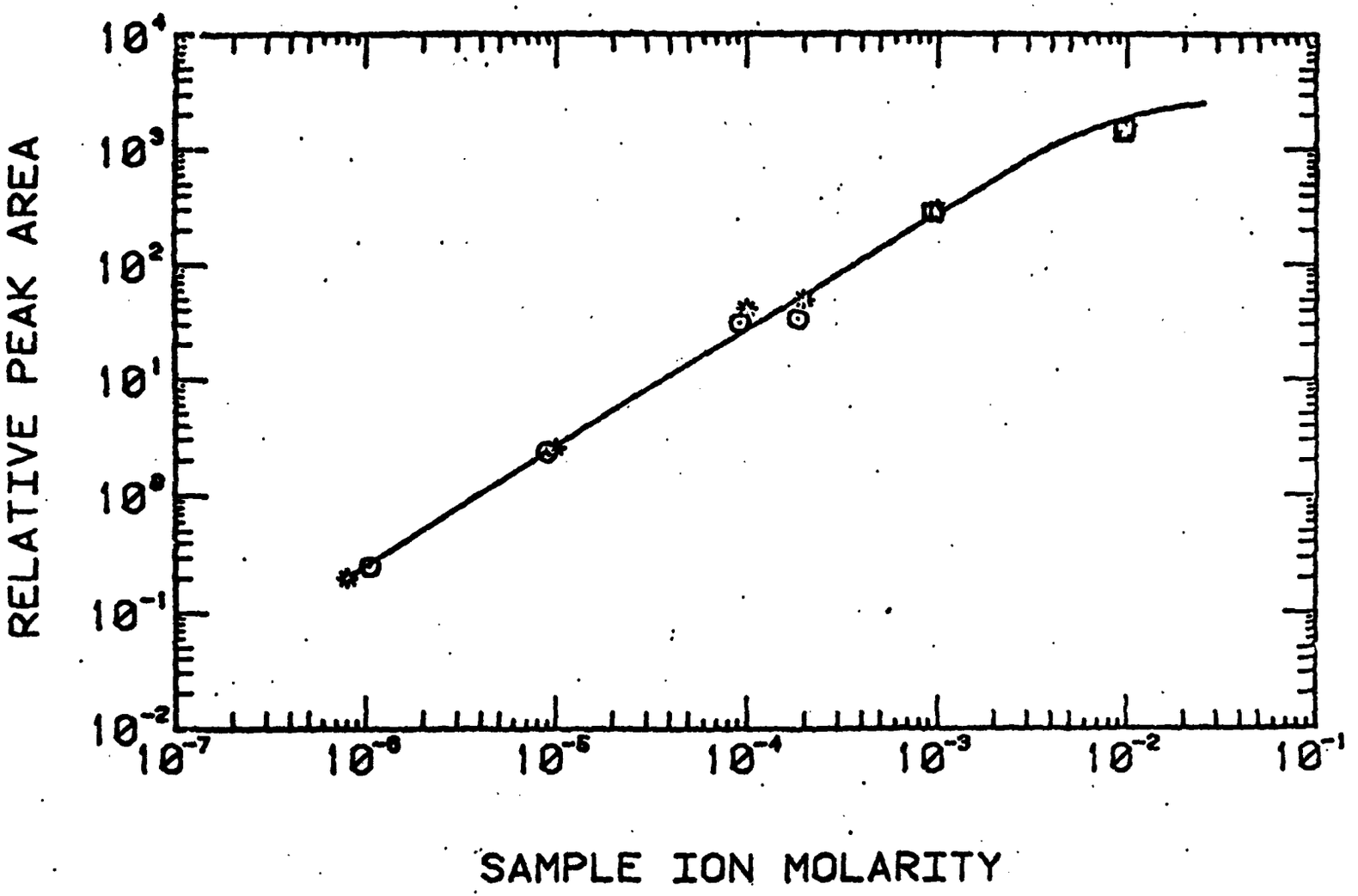
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Fig. 2

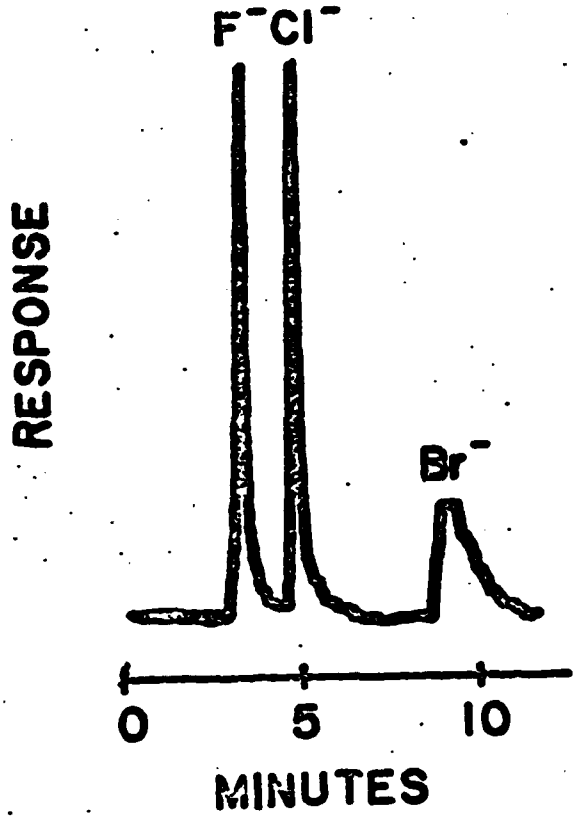


Fig. 3

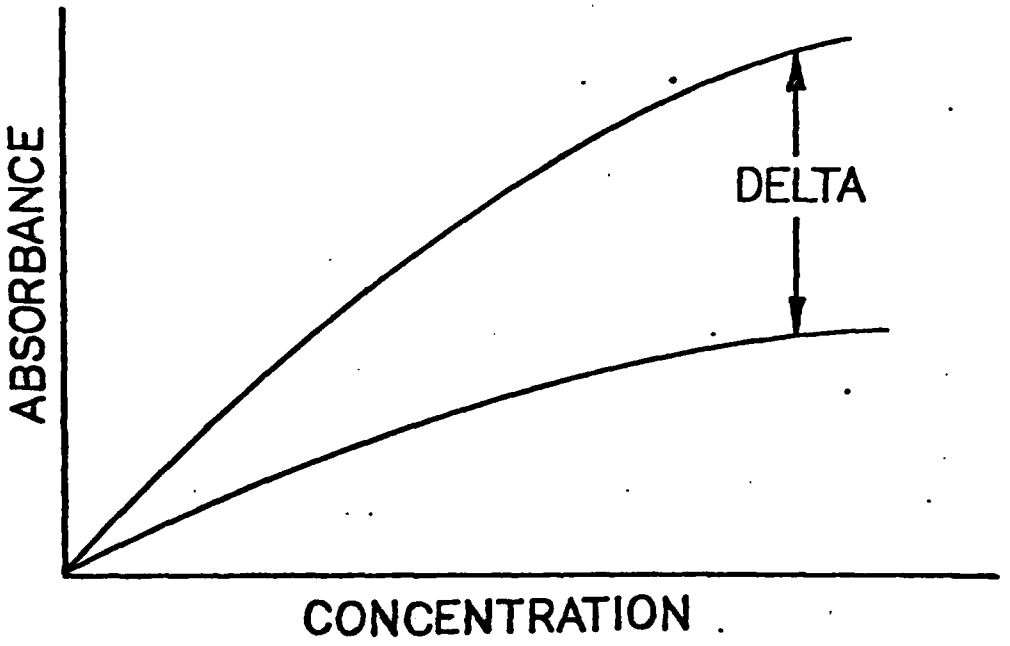


Fig. 4

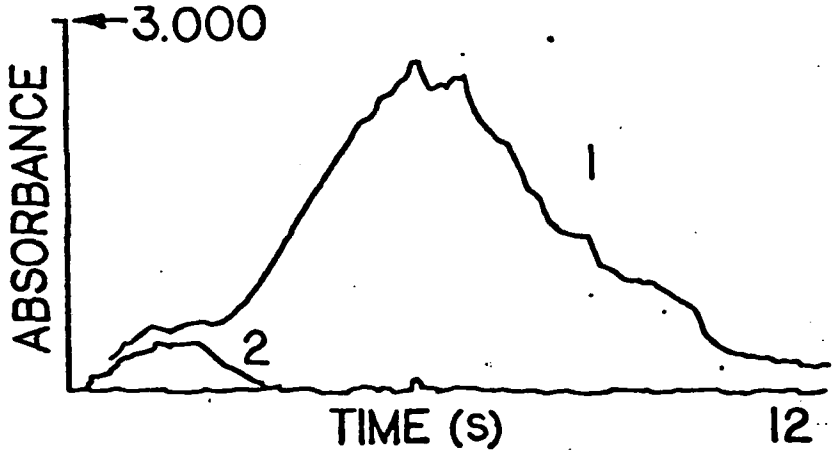


Fig. 5

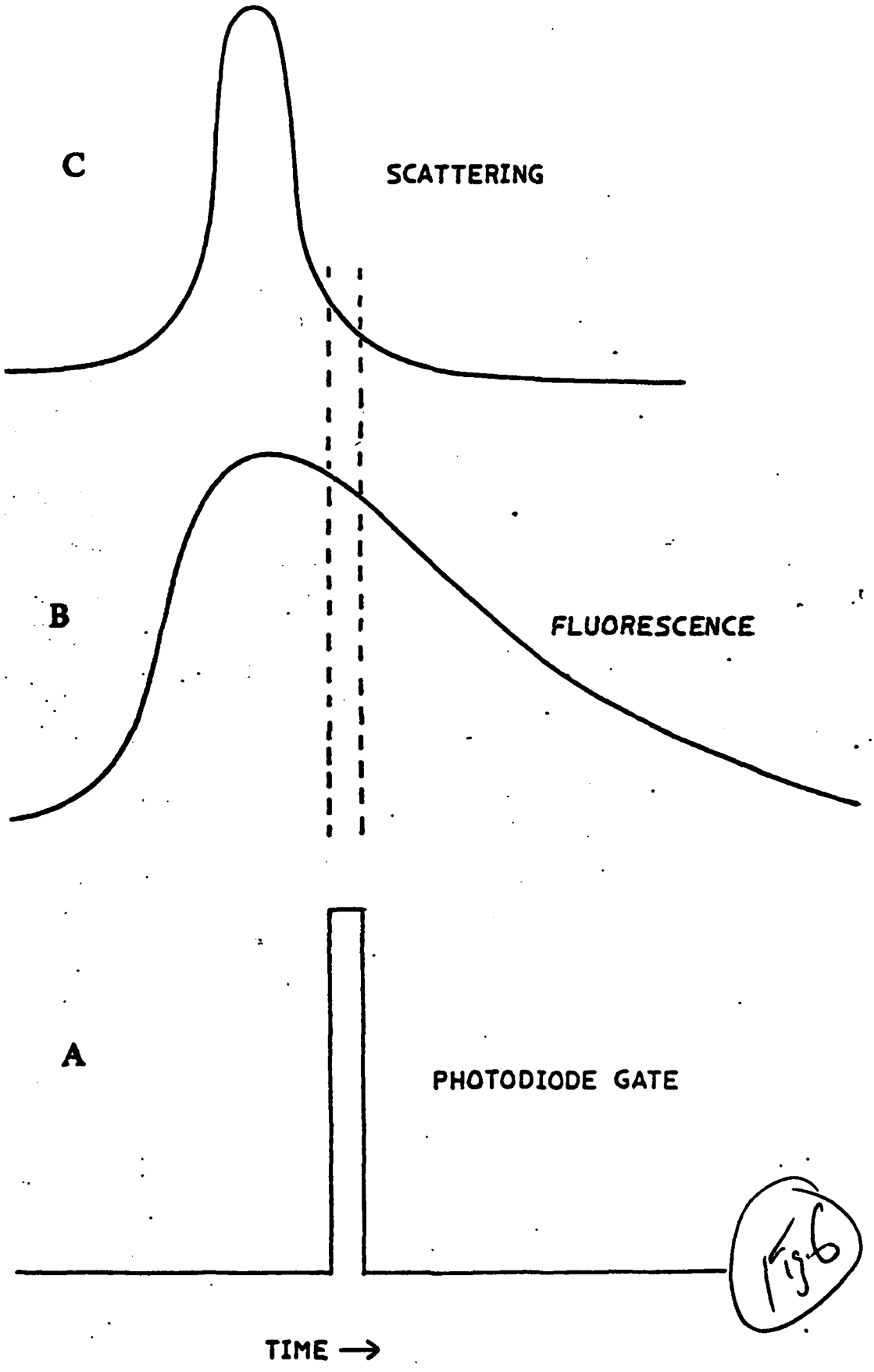
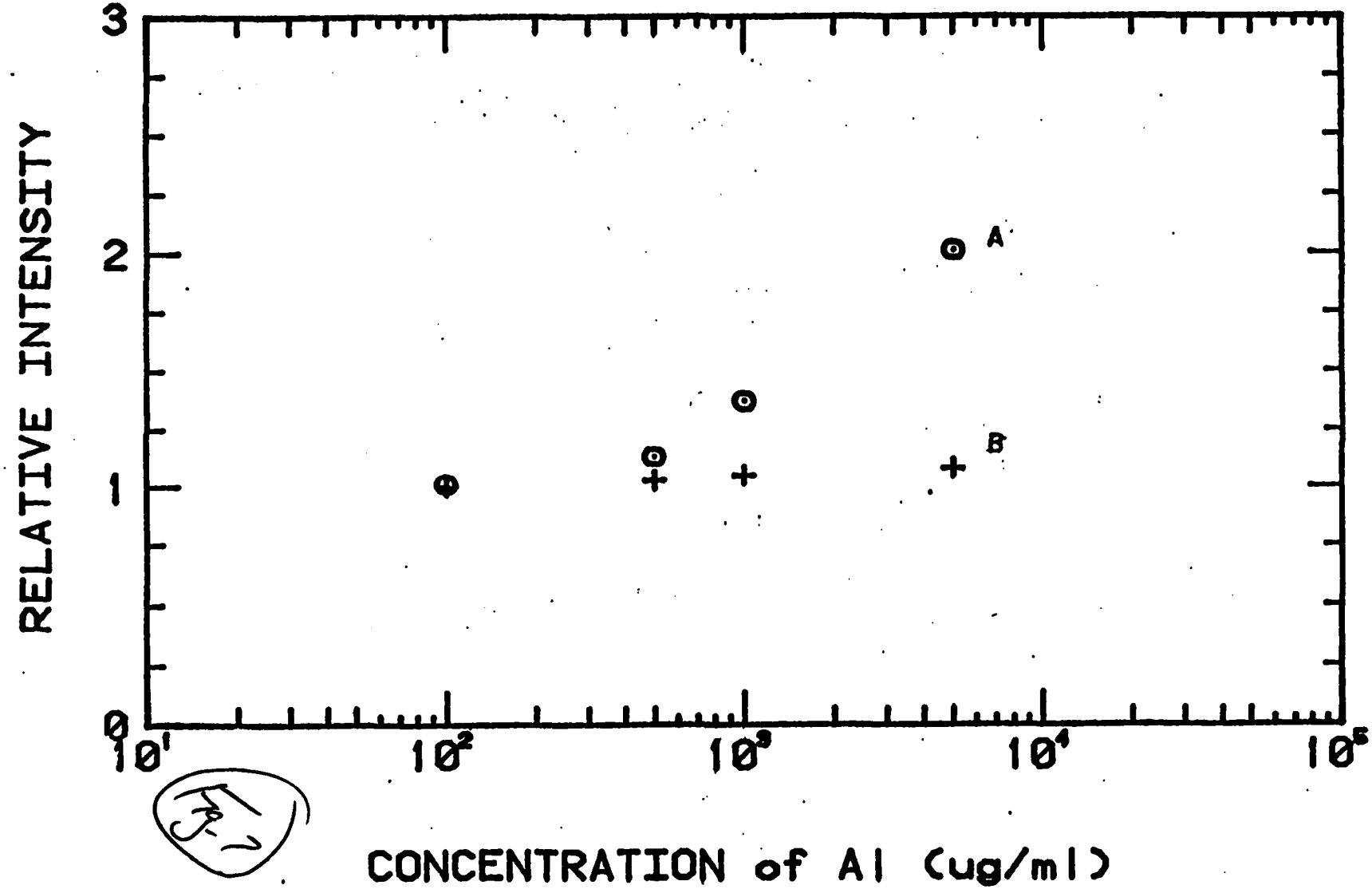


Fig 6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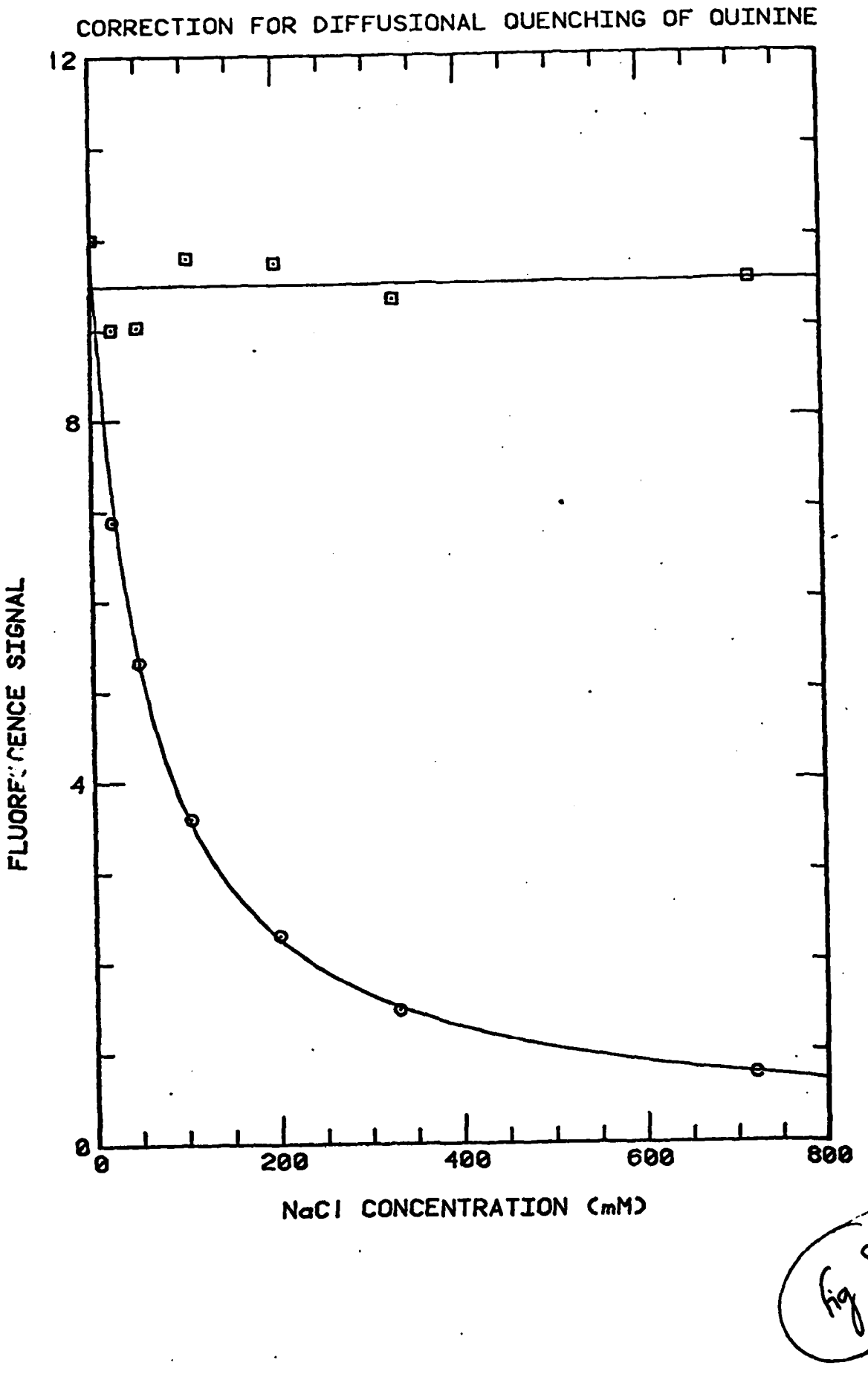
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NaCl CONCENTRATION (mM)

fig 8

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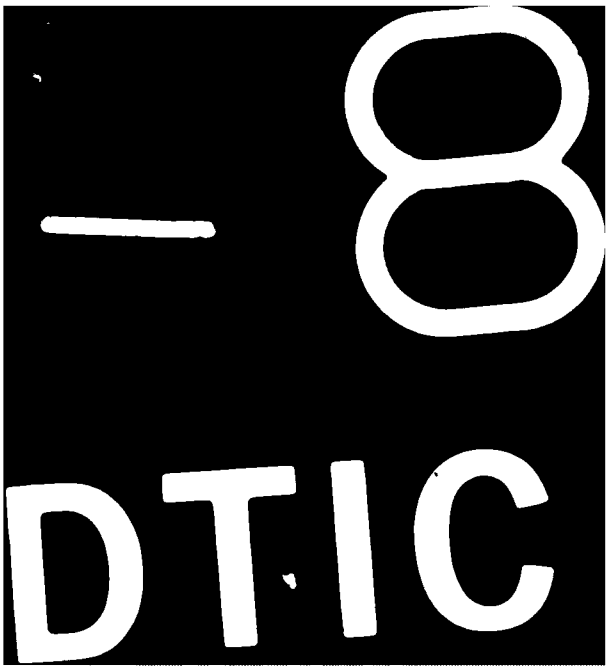
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